

Alterations of the Cell-Cycle Inhibitors p27^{KIP1} and p16^{INK4a} Are Frequent in Blastic Plasmacytoid Dendritic Cell Neoplasms

Thomas Wiesner^{1,4}, Anna C. Obenaus^{2,4}, Carlo Cota³, Isabella Fried¹, Michael R. Speicher² and Lorenzo Cerroni¹

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare, clinically aggressive malignancy with a median survival of 12–14 months. To identify pathogenetic relevant genomic aberrations and molecular targets for therapy, we analyzed skin biopsy samples obtained from 14 patients using high-resolution array-based comparative genomic hybridization and immunostaining. Losses of chromosomes 9, 12, 13, and 15 were detected most frequently. Loss of the *CDKN1B* locus was the most common finding and was detected in 64% of tumors. In all but one case, the dose-dependent haploinsufficient cell-cycle inhibitor p27^{KIP1}, encoded by *CDKN1B*, was weakly expressed in the nuclei of tumor cells. Losses of the *CDKN2A-ARF-CDKN2B* locus occurred in 50% of patients, and in one case a distinct biallelic loss was identified. The cell-cycle inhibitor p16^{INK4a}, which is encoded by *CDKN2A*, was not expressed in tumor cells, suggesting a complete loss of function. Loss of chromosome 13, including the *RB1* gene, was observed in 43% of tumors. These results imply that alterations of the cell-cycle checkpoint controlling proteins p27^{KIP1}, p16^{INK4a}, and *RB1* may exert a profound effect in malignant transformation in BPDCN. The elucidation of the affected pathways may guide the development of new treatments specifically designed for this aggressive disease entity.

Journal of Investigative Dermatology (2010) **130**, 1152–1157; doi:10.1038/jid.2009.369; published online 19 November 2009

INTRODUCTION

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare malignant proliferation derived from precursors of plasmacytoid dendritic cells. Clinically, patients usually present with reddish-brown (“bruise-like”), asymptomatic skin plaques and tumors, often followed by bone marrow, lymph node, and blood involvement. Although most cases show an initial response to multiagent chemotherapy, relapses are frequent and the clinical course is aggressive with a median survival of 12–14 months (Facchetti *et al.*, 2008).

Genetic aberrations have only been studied in a limited number of cases. Using techniques such as karyotyping, fluorescence *in situ* hybridization, or low-resolution comparative genomic hybridization (CGH), chromosomal abnormalities have been detected in 70% of cases. In these studies, alterations of chromosome regions 5q, 6q, 9, 12p,

13q, and 15q were most commonly found (Petrella *et al.*, 1999; Leroux *et al.*, 2002; Mao *et al.*, 2003; Reichard *et al.*, 2005). A combined array-CGH and gene-expression study of five cases showed recurrent deletions of chromosomal regions 4q34, 9, and 13q12–q31. In this study, the RNA expression of the tumor suppressor genes *RB1* and *LATS2* was decreased, whereas the expression of oncogenes *HES6*, *RUNX2*, and *FLT3* was increased (Dijkman *et al.*, 2007). Recently, an array-CGH study analyzed nine blood or lymph node samples of BPDCN and reported deletions of the tumor suppressor genes *RB1*, *CDKN1B*, *CDKN2A*, and *TP53* (Jardin *et al.*, 2009). The authors suggested that the loss of these genes may represent crucial oncogenetic events in BPDCN, because they may alter the G1/S transition.

Although studies by Dijkman *et al.* and Jardin *et al.* nicely demonstrated the power of array CGH to characterize genomic alterations in BPDCN, genetic data for this disease still remain limited and variable. In this study, we significantly extend the genetic data of BPDCN in a series of 14 patients and confirm the central genetic results on the protein level. Owing to the aggressive clinical course and the currently insufficient treatment options, we discuss possible implications of the revealed alterations for new therapeutic strategies.

RESULTS AND DISCUSSION

Patients and recurrent genomic changes

All 14 patients presented with typical clinical and histological features (1 woman, 13 men; aged 38–89 years; mean age

¹Research Unit of Dermatopathology, Department of Dermatology, Medical University of Graz, Graz, Austria; ²Institute of Human Genetics, Medical University of Graz, Graz, Austria and ³San Gallicano Dermatological Institute, Rome, Italy

⁴These authors contributed equally to this work.

Correspondence: Lorenzo Cerroni, Department of Dermatology, Medical University of Graz, Auenbruggerplatz 8, 8036 Graz, Austria.
E-mail: lorenzo.cerroni@medunigraz.at

Abbreviations: BPDCN, blastic plasmacytoid dendritic cell neoplasm; CGH, comparative genomic hybridization

Received 28 July 2009; revised 20 September 2009; accepted 13 October 2009; published online 19 November 2009

Table 1. Clinical and immunohistological features of our patients

Case	Age, years	Sex	Skin lesions at diagnosis	Extracutaneous disease at presentation	Follow-up, months	p27	p16
1	65	M	Generalized	No	A++, 6	±	–
2	73	M	Generalized	Yes	A++, 12	±	–
3	74	M	Localized	No	A+, 26	±	–
4	58	F	Localized	No	A++, 22	±	–
5	76	M	Generalized	No	NA	±	–
6	89	M	Generalized	Yes	DoD, 2	–	– ¹
7	77	M	Generalized	No	NA	±	–
8	84	M	Generalized	Yes	DoD, 9	±	–
9	50	M	Generalized	Yes	NA	±	–
10	38	M	Localized	No	CR, 12	±	–
11	42	M	Generalized	NA	DoD, 1	±	–
12	87	M	Localized	No	NA	±	–
13	80	M	Generalized	No	DoD, 2	±	–
14	57	M	Generalized	No	A+, 3	±	–

Abbreviations: A+ alive with skin disease; A++ alive with skin and systemic disease; CR, complete remission; DoD, died of disease; F, female; M, male; NA, not available; ±, predominant weak nuclear staining with scattered cells with stronger signal.

¹Scattered positive; –, negative.

68 years; Table 1). Only pretreatment skin biopsy samples were analyzed using genome-wide, high-resolution array CGH. Chromosomal aberrations, predominantly losses, were observed in all analyzed samples. Losses on chromosomes 12 (8 cases), 9 (7 cases), 13 (6 cases), and 15 (5 cases) were detected most frequently (Figure 1, Table 2). Analysis of available follow-up data did not show any correlation between specific sets of genetic aberrations or p27^{KIP1} and p16^{INK4a} immunohistochemical staining patterns and prognosis (Table 1). A complete summary of genomic aberrations of each case, including ideograms, start and stop positions of deleted/duplicated regions, and affected genes is included in Supplementary Table S1.

Loss of chromosome region 12p13 and the weak expression of p27^{KIP1}

Loss of the chromosome region 12p13 was the most frequent finding (Figure 2a). The common deleted region had a size of only 1.6 Mb and includes the *CDKN1B* gene, which encodes the p27^{KIP1} protein. To further confirm the involvement of this gene in oncogenesis, we performed immunostaining and found in all but 1 of our 14 cases, a weak expression of p27^{KIP1} in the nuclei of tumor cells (Figure 2c). Between these weakly p27^{KIP1}-expressing cells, few scattered tumor cells with a strong nuclear expression were constantly found. The monoallelic losses of *CDKN1B* in 64% of cases and the weak nuclear expression of p27^{KIP1} in the great majority of tumor cells indicate the presence of an unaltered second allele.

The p27^{KIP1} protein is an important dose-dependent haploinsufficient tumor suppressor and has complex functions in cell-cycle regulation (Chu *et al.*, 2008). High levels of p27^{KIP1} arrest the cell cycle by inhibiting cyclin E-dependent kinases, but low levels of p27^{KIP1} drive the cell cycle by stabilizing cyclin D-dependent kinases (Sherr and Roberts,

2004). Inactivation of both p27^{KIP1} alleles is rare in tumors, possibly because it may not provide sufficient advantage over the heterozygous state (Fero *et al.*, 1998). In most human malignancies, including lung, breast, ovarian, prostatic, gastric, and colon cancer, deletions and mutations of *CDKN1B* are rare, but downregulation of p27^{KIP1} due to other mechanisms is common and correlates strongly with high tumor grade and poor prognosis (Chu *et al.*, 2008). The frequent loss of the *CDKN1B* gene in BPDCN, and the constantly weak expression of its gene product p27^{KIP1}, may indicate a profound effect on tumorigenesis, and may partly explain the aggressiveness of this disease.

The commonly deleted 12p13 region also harbors the *ETV6* gene, which acts as a transcriptional repressor. In hematological malignancies, alterations of both *ETV6* alleles are frequently observed; one allele is involved in a translocation resulting in the formation of a new fusion gene, whereas the second allele is lost (Bohlander, 2005). These concomitant translocations and deletions led to the suggestion that *ETV6* may act as a tumor suppressor, but the oncogenetic relevance seems to result more from the formation of fusion genes than from deletions (Bohlander, 2005). However, translocations involving *ETV6* have not yet been reported by karyotyping of BPDCN (Petrella *et al.*, 1999; Leroux *et al.*, 2002; Reichard *et al.*, 2005), and the relevance of the *ETV6* loss alone is at present unclear.

Loss of chromosome 9 and no expression of p16^{INK4a}

Chromosome 9 was the second most commonly affected in our series. Interestingly, in one case, we found a small biallelic deletion of only 500 kb (Figure 2b), harboring the *CDKN2A-ARF-CDKN2B* locus, which encodes the two inhibitors of cyclin D-dependent kinases, p15^{INK4b} and p16^{INK4a}, and the unrelated *ARF* protein. In all of our cases,

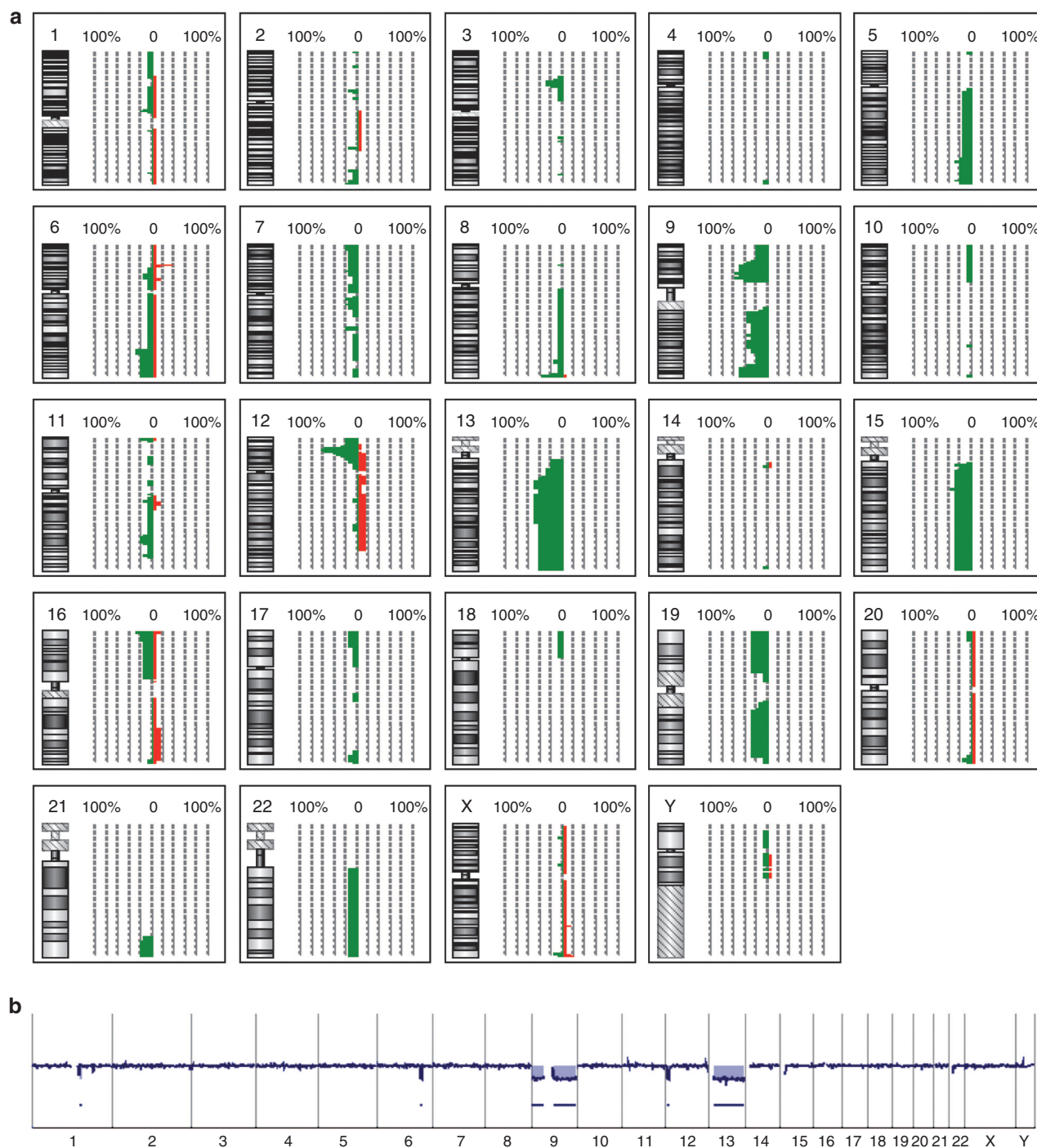


Figure 1. Genomic aberrations in BPDCN detected by array CGH. (a) The ideogram summarizes chromosomal gains and losses of 14 patients. The green and red bars on the right side of each chromosome indicate how often, that is, in what percentage, copy number changes were found. Losses are displayed in green in the left half of each figure, and gains are shown in red in the right half. Deletions at chromosomes 9, 12, 13, 15, and 19 were most often observed, whereas other changes only occurred in a low percentage of cases. **(b)** Representative genome-wide array-CGH profile of case 1. On the x axis, the data are organized along the 22 autosomal chromosomes and the 2 sex chromosomes. The y axis shows the relative copy number on a logarithmic scale. The blue bars define losses on chromosome 1, 6, 9, 12, 13. Abbreviations: BPDCN, blastic plasmacytoid dendritic cell neoplasm; CGH, comparative genomic hybridization.

immunostaining showed that p16^{INK4a} was indeed not expressed in tumor cells (Figure 2d). The losses of the *CDKN2A-ARF-CDKN2B* locus in 50% of our patients, and

the biallelic loss in one of them, as well as the constantly negative expression of p16^{INK4a} is highly suggestive of a complete loss of function of p16^{INK4a}. In addition to the

Table 2. Copy number changes and candidate genes detected in more than 20% of cases

Chromosome region	Affected patients, %	Change	Candidate genes
12p13.2	64	Loss	<i>CDKN1B</i> , <i>ETV6</i>
9p21.3, 9q34	50	Loss	<i>CDKN2A</i> , <i>CDKN2B</i> , <i>ARF</i> ¹
13q12.11–q34	43	Loss	<i>RB1</i>
15q11.2–q26.3	36	Loss	²
19p13.3–p13.4	29	Loss	²
3p22.2–p21.1	29	Loss	<i>PTPN23</i>
5q32–q35.2	21	Loss	²
6q23.3–q27	21	Loss	<i>PARK2</i>
7p22.3–p22.1	21	Loss	<i>MAD1L1</i>
21q22.3	21	Loss	²

Abbreviation: BPDCN, blastic plasmacytoid dendritic cell neoplasm.
¹In addition to the *CDKN2A-ARF-CDKN2B* locus, losses on chromosome 9 included large parts of the chromosome and several additional candidate genes possible involved in the pathogenesis of BPDCN.
²Candidate genes are not mentioned, because losses included large parts of chromosomes and hundreds of genes. For all cases, a list of genomic aberrations including start and stop position, cytogenetic bands, and fluorescence log2 ratio are included in Supplementary Table S1.

observed losses of the *CDKN2A-ARF-CDKN2B* locus, other mechanisms such as mutations or epigenetic modifications may affect the undeleted p16^{INK4a} alleles.

Gene products of the *CDKN2A-ARF-CDKN2B* locus fulfill the classical Knudson criteria for tumor suppression (“2-hit” model) and participate in major tumor suppressor networks that are disabled in human cancer. The p16^{INK4a} and p15^{INK4b} proteins both arrest the cell cycle by inhibiting the cyclin D-dependent-kinases-mediated phosphorylation of *RB1* (Kim and Sharpless, 2006). *ARF* inhibits the degradation of the p53 protein (Kim and Sharpless, 2006). Losses of these proteins in BPDCN harmfully deregulate both the *RB1* and p53 pathways, indicating a critical involvement in the pathogenesis of this tumor entity.

Although losses of the *CDKN2A-ARF-CDKN2B* locus were also reported by Jardin *et al.*, we additionally found frequent losses (≥50%) in chromosomal regions 9p21.1, 9p13.3, and 9q34.2. This suggests that other regions and their respective genes on chromosome 9 may be involved in BPDCN pathogenesis.

Losses of chromosome 13 and *RB1*

We observed chromosome 13 deletions in 43% of tumors, which involved large parts of the chromosome and several tumor suppressor genes. Among those, the *RB1* gene is most prominent and arrests the cell cycle by repressing the transcription of genes, the products of which are required for DNA synthesis. Cyclin D-dependent kinases, which are possibly hyperactive because of losses of their inhibitors p16^{INK4a} and p15^{INK4b}, phosphorylate *RB1* and cancel its growth-inhibitory effects. Jardin *et al.* (2009) suggested that *RB1* might be the target gene in this region, and Dijkman *et al.*

(2007) demonstrated a diminished gene expression of *RB1* using RNA microarray analyses of five BPDCNs. A profound effect of *RB1* on tumorigenesis of BPDCN seems likely.

Losses on chromosomes 5q, 9, 12, 13, and 15 are most common

Our results significantly extend the current knowledge regarding genetic changes in BPDCN. The three largest previous studies altogether comprised data on 27 patients (Leroux *et al.*, 2002; Dijkman *et al.*, 2007; Jardin *et al.*, 2009). Together with the 14 new cases described in this study, a relatively homogeneous pattern of common alterations in this tumor entity emerges. Losses of the common deleted region on chromosomes 9, 12, and 15 were detected in our patients and in the 3 studies in ~50, 60, and 30% of cases, respectively. Losses of chromosome 13 and 5q were found in our patients in a lower percentage than in the pooled data set (43 vs 73% and 21 vs 48%, respectively). Other genomic aberrations such as losses at 6q (Leroux *et al.*, 2002), 4q (Dijkman *et al.*, 2007), or 17p (Jardin *et al.*, 2009), were found inconsistently in the three studies, and were also uncommon in our patients.

Conclusions and possible therapeutic implications

Our data are in agreement with previous notions by Jardin *et al.* that the most frequently observed deletions in the BPDCN tumor genomes most likely affect the G1/S transition. The alteration of the important cell-cycle regulating genes, namely *CDKN1B*, *CDKN2A*, *ARF*, *CDKN2B*, and *RB1*, may contribute to overactive cell-cycle kinases, which is often associated with aberrant division and uncontrolled proliferation of cancer cells.

The elucidation of the affected pathways in this disease may guide the development of new treatment options, specifically designed for this tumor entity. For example, in this study, we demonstrate the downregulation of p27^{KIP1}, which likely destabilizes cell-cycle control. Restoration of normal p27^{KIP1} levels may represent an efficient treatment option and could possibly be achieved by treatment with the mammalian target of rapamycin inhibitors (Chu *et al.*, 2008; Meric-Bernstam and Gonzalez-Angulo, 2009). Inhibitors of mammalian target of rapamycin induce cell-cycle arrests in various cell systems by inactivating cyclin D-dependent kinases, which are believed to result from the downregulation of D-type cyclins and accumulation of p27^{KIP1}. Moreover, the downregulation of D-type cyclins may also reduce the negative impact of p16^{INK4a} and p15^{INK4b} losses on the cell cycle. Clinical trials using mammalian target of rapamycin inhibitors have already shown promising results and clinical benefits in several cancer types, including lymphoma. On the basis of the genetic and immunostaining results, it seems possible that mammalian target of rapamycin inhibitors may be effective in BPDCN, and it seems worth evaluating their efficacy in preclinical and clinical settings.

MATERIALS AND METHODS

Tumor samples and immunophenotyping

The study was approved by the institutional ethics committee and was conducted according to the Declaration of Helsinki. The tumor

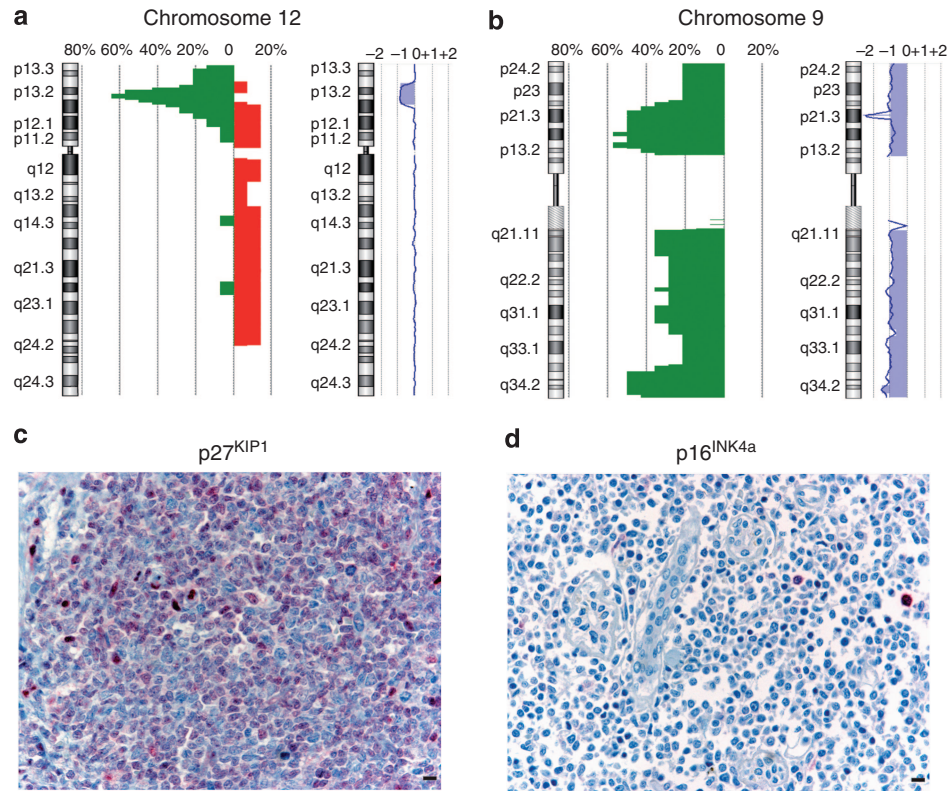


Figure 2. Losses of chromosomes 9 and 12 in detail. (a) The common deleted region on chromosome 12, detected in 64% of cases, is only 1.6 Mb large, and contains the *CDKN1B* gene. The right part of this figure shows the array-CGH profile of case 1. The blue line follows a log ratio of 0 over large parts of chromosome 12, indicating a normal copy number, but becomes negative at 12p13 (log ratio of -1), pointing at a monoallelic loss of this region. The shaded blue area defines the lost region. (b) Losses on chromosome 9 were detected in 50% of cases. The right part of this figure illustrates a biallelic, 500 kb sized deletion at 9p21.3 (case 8). The blue line shows the log ratio and indicates at -1 , a monoallelic loss of the whole chromosome 9; values < -2 indicate an additional loss of the *CDKN2A-ARF-CDKN2B* locus of the second chromosome. (c) Immunostaining of case 8 shows a weak expression of p27^{KIP1} in the nuclei of tumor cells, but also scattered admixed malignant cells with a strong nuclear p27^{KIP1} expression. Bar = 10 μ m. (d) Immunostaining for p16^{INK4a} was constantly negative as shown herein in case 7. Bar = 10 μ m. Abbreviation: CGH, comparative genomic hybridization.

samples were formalin-fixed, paraffin-embedded tissue archival specimens (12 cases) or flash-frozen tissues (2 cases). The diagnosis of BPDCN was made by two independent dermatopathologists with appropriate immunohistochemical stainings as described previously (Ascani *et al.*, 2008; Facchetti *et al.*, 2008). The protein expression of p27^{KIP1} and p16^{INK4a} was evaluated with a standard immunoperoxidase technique using appropriate antibodies (p27^{KIP1}: Dako, Glostrup, Denmark; p16^{INK4a}: MTM, Heidelberg, Germany).

DNA extraction

For DNA extraction, 10- μ m sections were cut, and only areas with a tumor cell infiltration of more than 90% were microdissected as determined by a microscopic examination of adjacent sections. DNA was purified using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany).

Array CGH

Samples were labeled using a Bioprime Array CGH Genomic Labeling Kit according to the manufacturer's instructions (Invitrogen, Carlsberg, CA). Briefly, 500 ng test DNA and reference DNA (Promega, Madison, WI) were differentially labeled with dCTP-Cy5 and dCTP-Cy3, respectively (GE Healthcare, Piscataway, NJ).

Genome-wide analysis of DNA copy number changes was conducted using an oligonucleotide array containing 44,000 probes with a spatial resolution of 35 kb according to the manufacturer's protocol version 6.0 (Agilent, Santa Clara, CA). Slides were scanned using Agilent's microarray scanner G2505B and analyzed using Agilent DNA Analytics software 4.0.76 (statistical algorithm: ADM-2; sensitivity threshold: 8.0; consecutive clone filter: 10).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the Jubiläumsfonds of the Oesterreichische Nationalbank (OeNB) grant 12480. Anna C Obenauf was funded by the Molecular Medicine PhD program of the Medical University of Graz. We are indebted to Ulrike Schmidbauer for her excellent technical assistance.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

REFERENCES

- Ascani S, Massone C, Ferrara G *et al.* (2008) CD4-negative variant of CD4+/CD56+ hematodermic neoplasm: description of three cases. *J Cutan Pathol* 35:911–5
- Bohlander SK (2005) ETV6: a versatile player in leukemogenesis. *Semin Cancer Biol* 15:162–74
- Chu I, Hengst L, Slingerland J (2008) The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. *Nat Rev Cancer* 8:253–67
- Dijkman R, Van Doorn R, Szuhai K *et al.* (2007) Gene-expression profiling and array-based CGH classify CD4+CD56+ hematodermic neoplasm and cutaneous myelomonocytic leukemia as distinct disease entities. *Blood* 109:1720–7
- Facchetti F, Jones D, Petrella T (2008) Blastic plasmacytoid dendritic cell neoplasms. In: *WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues*. (Swerdlow SH, Campo E, Harries NL, Jaffe ES, Pileri SA, Stein H, *et al.*, eds) Lyon: IARC. pp 145–7
- Fero ML, Randel E, Gurley KE *et al.* (1998) The murine gene p27Kip1 is haplo-insufficient for tumour suppression. *Nature* 396:177–80
- Jardin F, Callanan M, Penther D *et al.* (2009) Recurrent genomic aberrations combined with deletions of various tumour suppressor genes may deregulate the G1/S transition in CD4+CD56+ haematodermic neoplasms and contribute to the aggressiveness of the disease. *Leukemia* 23:698–707
- Kim WY, Sharpless NE (2006) The regulation of INK4/ARF in cancer and aging. *Cell* 127:265–75
- Leroux D, Mugneret F, Callanan M *et al.* (2002) CD4(+), CD56(+) DC2 acute leukemia is characterized by recurrent clonal chromosomal changes affecting 6 major targets: a study of 21 cases by the Groupe Français de Cytogénétique Hématologique. *Blood* 99:4154–9
- Mao X, Onadim Z, Price EA *et al.* (2003) Genomic alterations in blastic natural killer/extranodal natural killer-like T cell lymphoma with cutaneous involvement. *J Invest Dermatol* 121:618–27
- Meric-Bernstam F, Gonzalez-Angulo A (2009) Targeting the mTOR signaling network for cancer therapy. *J Clin Oncol* 27:2278–87
- Petrella T, Dalac S, Maynadié M *et al.* (1999) CD4+ CD56+ cutaneous neoplasms: a distinct hematological entity? Groupe Français d'Etude des Lymphomes Cutanés (GFELC). *Am J Surg Pathol* 23:137–46
- Reichard KK, Burks EJ, Foucar MK *et al.* (2005) CD4(+) CD56(+) lineage-negative malignancies are rare tumors of plasmacytoid dendritic cells. *Am J Surg Pathol* 29:1274–83
- Sherr C, Roberts JM (2004) Living with or without cyclins and cyclin-dependent kinases. *Genes Dev* 18:2699–711